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RECEIVED 19 January 2023

ACCEPTED 05 April 2023

PUBLISHED 27 April 2023

CITATION

Zelendova M, Papagiannitsis CC, Sismova P, Medvecký M, Pomorska K, Palkovicova J, Nesporova K, Jakubu V, Jamborova I, Zemlickova H, Dolejska M and Working Group for Monitoring of Antibiotic Resistance (2023) Plasmid-mediated colistin resistance among human clinical *Enterobacterales* isolates: national surveillance in the Czech Republic. *Front. Microbiol.* 14:1147846. doi: 10.3389/fmicb.2023.1147846

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Plasmid-mediated colistin resistance among human clinical *Enterobacterales* isolates: national surveillance in the Czech Republic

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The occurrence of colistin resistance has increased rapidly among *Enterobacterales* around the world. We performed a national survey of plasmid-mediated colistin resistance in human clinical isolates through a retrospective analysis of samples from 2009 to 2017 and a prospective sampling in 2018–2020. The aim of this study was to identify and characterize isolates with *mcr* genes from various regions of the Czech Republic using whole genome sequencing (WGS). Of all 1932 colistin-resistant isolates analyzed, 73 (3.8%) were positive for *mcr* genes. Most isolates carried *mcr-1* (48/73) and were identified as *Escherichia coli* ($n=44$) and *Klebsiella pneumoniae* ($n=4$) of various sequence types (ST). Twenty-five isolates, including *Enterobacter* spp. ($n=24$) and *Citrobacter freundii* ($n=1$) carrying the *mcr-9* gene were detected; three of them (*Enterobacter kobei* ST54) co-harbored the *mcr-4* and *mcr-9* genes. Multi-drug resistance phenotype was a common feature of *mcr* isolates and 14% (10/73) isolates also co-harbored clinically important beta-lactamases, including two isolates with carbapenemases KPC-2 and OXA-48. Phylogenetic analysis of *E. coli* ST744, the dominant genotype in this study, with the global collection showed Czech isolates belonged to two major clades, one containing isolates from Europe, while the second composed of isolates from diverse geographical areas. The *mcr-1* gene was carried by IncX4 (34/73, 47%), IncHI2/ST4 (6/73, 8%) and IncI2 (8/73, 11%) plasmid groups. Small plasmids belonging to the ColE10 group were associated with *mcr-4* in three isolates, while *mcr-9* was carried by IncHI2/ST1 plasmids (4/73, 5%) or the chromosome (18/73, 25%). We showed an overall low level of occurrence of *mcr* genes in colistin-resistant bacteria from human clinical samples in the Czech Republic.

KEYWORDS

antibiotic resistance, *Enterobacterales*, human, *mcr*, plasmids

Introduction

The excessive consumption of antimicrobial substances associated with faster spread of antibiotic resistance represents a global concern. The dissemination of multi-drug resistant (MDR) bacteria resulted in limited treatment options of infectious diseases in healthcare systems. The interest in the administration of older antibiotics such as polymyxins has therefore been renewed (Bitar et al., 2020). Colistin has been widely used in the past, but due to its nephrotoxicity and neurotoxicity, it has become a restricted antibiotic (Viñes et al., 2021). Currently, colistin is administered for the treatment of life-threatening infections caused by MDR Gram-negative pathogens as the last-resort antibiotic (Yilmaz et al., 2016; Hamel et al., 2021; Viñes et al., 2021). In contrast, colistin has been widely used for prophylactic and therapeutic purposes in veterinary medicine for decades (Quiroga et al., 2019; Viñes et al., 2021). However, colistin overuse in livestock has led to the spread of colistin-resistant pathogens worldwide and the development of different strategies used by bacteria to increase resistance against colistin (El-Sayed Ahmed et al., 2020).

Resistance to colistin can be either associated with chromosomal mutations or with *mcr* genes carried by plasmids that are facilitating horizontal transfer of colistin resistance between bacteria (Zhu et al., 2019). Acquired colistin-resistance mechanisms have been recognized in some members of the Enterobacteriaceae family, such as *E. coli*, *Salmonella* spp., *Klebsiella* spp., and *Enterobacter* spp. These include genes and operons responsible for encoding enzymes that have a direct role in LPS modification, such as the *pmrC* and *pmrE* genes and the *pmrHFIJKLM* operon (Aghapour et al., 2019). Apart from the chromosomally-mediated mechanisms, 10 variants, *mcr-1* to *mcr-10*, carried by various plasmid families have been so far identified in *Enterobacteriales*, especially in *E. coli* and *Enterobacter* spp. (Bitar et al., 2020; Javed et al., 2020; Li et al., 2020; Wang C. et al., 2020). The most common variant, *mcr-1*, is usually located on plasmids of various incompatibility (Inc) groups, but predominantly on IncX4, IncI2 and IncHI2 (Carattoli et al., 2014; Doumith et al., 2016; Zelendova et al., 2021). These plasmid types carrying *mcr-1* were found in *Enterobacteriales* isolates from humans as well as farm animals around the globe (Dalmolin et al., 2018; Quiroga et al., 2019), highlighting their wide distribution in various niches. Besides *mcr-1*, other genes for plasmid-mediated colistin resistance, such as *mcr-4* and *mcr-9*, have been reported (Bitar et al., 2020; Li et al., 2020). The *mcr-4* gene is usually located on small ColE10-type plasmids (Carattoli et al., 2017; Marchetti et al., 2021) while *mcr-9* is mostly carried by large IncHI2 plasmids or is incorporated into the chromosome (Li et al., 2020; Tyson et al., 2020).

The emergence of colistin resistance in MDR bacteria is a significant clinical concern. Isolates encoding extended-spectrum beta-lactamase (ESBL) or carbapenemase on a single plasmid along with *mcr* have been detected (Carattoli et al., 2014; Katip et al., 2021). As the co-occurrence of more resistance genes within the bacteria represents a threat to current medicine, The European Centre for Disease Prevention and Control (ECDC) published the expert protocol that recommends performing the surveillance of co-resistance to both colistin and carbapenems in *Enterobacteriales* (ECDC, European Centre for Disease Prevention and Control, 2019).

From the Czech Republic, only sporadic reports describing the identification of *mcr*-carrying isolates in clinical samples have been published so far (Bitar et al., 2019; Bitar et al., 2020; Krutova et al.,

2021). However, overview data on the prevalence of *mcr* genes in Czech patients are not available. To fill in this gap, we aim to identify *mcr* genes in colistin-resistant human clinical isolates of Gram-negative bacteria from the Czech Republic between 2009 and 2020, and to determine characteristics of the *mcr*-positive strains using whole genome sequencing (WGS), plasmid typing and transferability experiments.

Materials and methods

Sampling and detection of *mcr* genes

A total of 1932 colistin-resistant isolates of Gram-negative bacteria with a minimum inhibitory concentration (MIC) to colistin >2 mg/L collected from Czech patients between 2009 and 2020 were examined. The collection consisted of 682 retrospective isolates obtained from 2009 through 2017 during various surveillance programmes at the National Institute of Public Health that were not targeting colistin resistance. The retrospective collection included mainly *Klebsiella pneumoniae* (*n* = 429), *Enterobacter* spp. (*n* = 108), *Pseudomonas aeruginosa* (*n* = 49), *Acinetobacter baumannii* (*n* = 33), *Stenotrophomonas maltophilia* (*n* = 20), *Escherichia coli* (*n* = 14) and 29 isolates of other 13 species. Prospective surveillance of colistin resistance in Czech hospitals was carried out during 2.5-year period between January 2018 and June 2020. It resulted in the collection of 1,250 isolates of *Klebsiella pneumoniae* (*n* = 491), *Enterobacter* spp. (*n* = 311), *Escherichia coli* (*n* = 179), *Pseudomonas aeruginosa* (*n* = 99), *Acinetobacter baumannii* (*n* = 43), *Hafnia alvei* (*n* = 28), *Klebsiella variicola* (*n* = 20), *Acinetobacter* spp. (*n* = 15), *Salmonella enterica* (*n* = 15), *Klebsiella oxytoca* (*n* = 14), *Klebsiella aerogenes* (*n* = 10), and 25 isolates of other 11 species. Strain identification was performed by a matrix-assisted laser desorption ionization-time of flight mass spectrometer (MALDI-TOF) using MALDI Biotyper software (Bruker Daltonics, Bremen, Germany). Species-level discrimination of *Enterobacter* spp. was performed using average nucleotide identity (ANI) (Yoon et al., 2017) and digital DNA-DNA hybridization (Meier-Kolthoff et al., 2013) of whole genome sequences. Eight types of strains were used as reference species, including *Enterobacter asburiae* (ATCC35953^T), *Enterobacter bugandensis* (DSM 29888^T), *Enterobacter cloacae* (ATCC13047^T), *Enterobacter dykesii* (DSM111347^T), *Enterobacter hormaechei* (ATCC49162^T), *Enterobacter kobei* (DSM13645^T), *Enterobacter vonholyi* (DSM110788^{TT}), *Enterobacter roggenkampii* (DSM16690^T).

All isolates were subjected to multiplex polymerase chain reaction (PCR) to detect the variants of *mcr* genes (*mcr-1* to *mcr-9*) (Rebelo et al., 2018; Wang et al., 2018; Kieffer et al., 2019).

Antimicrobial susceptibility testing

Susceptibility profiles of *mcr*-positive isolates were determined by the broth microdilution method using the following 15 antimicrobial substances: amikacin, ampicillin, ampicillin/sulbactam, cefepime, cefotaxime, cefoxitin, ceftazidime, ceftolozane/tazobactam, colistin, cotrimoxazole, ciprofloxacin, gentamicin, meropenem, piperacillin/tazobactam and tobramycin. The production of ESBL and AmpC type beta-lactamase was tested by a double-disk synergy test (European

Committee on Antimicrobial Susceptibility Testing, 2017). The production of carbapenemase was tested by a combination disc test method (European Committee on Antimicrobial Susceptibility Testing, 2017) and biochemical tests (BioRad-Beta-Carba test), while carbapenem hydrolysis was tested by MALDI-TOF (Papagiannitsis et al., 2015).

Conjugative transfer of *mcr* genes

Conjugation assays were performed to determine the transferability of *mcr* genes into plasmid-free sodium azide-resistant *E. coli* J53 K12 recipient cells using the filter-mating method (Borowiak et al., 2019). The transconjugants (TCs) were selected on LB agar plates (LBA) with sodium azide (100 mg/L) and colistin (0.5 mg/L). Successful transfer of the plasmid-mediated colistin resistance *via* conjugation was confirmed by PCR targeting the *mcr* gene (Rebello et al., 2018; Wang et al., 2018; Kieffer et al., 2019) and *E. coli* J53 K12 (Bauer et al., 2007). The size and number of plasmids transferred were estimated by pulsed-field gel electrophoresis (PFGE) using S1 nuclease (Centers for Disease Control and Prevention, 2004) and PCR-based replicon typing (PBRT; Carattoli et al., 2005).

Whole genome sequencing and plasmid characterization

Genomic DNA of all *mcr*-positive isolates was extracted using the NucleoSpin® Tissue Kit (Macherey-Nagel GmbH & Co, Duren, Germany). The libraries were prepared using the Nextera XT DNA Sample Preparation Kit and sequenced on the MiSeq or NovaSeq 6,000 platform (Illumina, San Diego, CA, United States). Raw reads were quality- and adaptor-trimmed using Trimmomatic v0.39 (Bolger et al., 2014) and assembly was performed by SPAdes v3.12.0 (Bankevich et al., 2012) and assembled data were analyzed using the CGE tools¹ that were used to identify antibiotic resistance genes (ResFinder 4.1) (Zankari et al., 2012), multi-locus sequence types (MLST 2.0) (Larsen et al., 2012), plasmid replicons (PlasmidFinder 2.1) and plasmid sequence types (STs) (pMLST 2.0) (Carattoli et al., 2014). Chromosomal mutations for resistance to fluoroquinolones and colistin in *E. coli* and *K. pneumoniae* isolates were determined by PointFinder (Zankari et al., 2017). Sequences of six IncX4 plasmids carrying *mcr-1* were extracted from Illumina data and gaps were filled by a PCR-based strategy and Sanger sequencing.

A complete nucleotide sequence of 12 selected isolates was obtained using long-read sequencing on the MinION platform (Oxford Nanopore Technologies, ONT, Oxford, United Kingdom). Genomic DNA was extracted by Genfind V3 (Beckman Coulter, United States). Libraries were constructed using a SQK-RBK004 rapid barcoding 1D kit according to the manufacturer's protocol. The barcoded library mix was loaded onto a flow cell (FLO-MIN106 R9.4 SpotON) and sequenced for 48 h. The raw electrical signals were basecalled using Guppy v4.2.2 (ONT) and raw reads in fastq format

were obtained. BBDuk² and Porechop v0.2.4 (ONT) were used for adaptor and quality trimming ($Q \leq 7$) and for demultiplexing, respectively. Whole plasmid sequences were assembled using Unicycler v0.4.8 (Wick et al., 2017) and Flye v2.6 (Lin et al., 2016) and polished by Illumina reads using Pilon v1.23 (Walker et al., 2014). For sequence analysis and annotation, BLAST³, the ISfinder database, and the open reading frame (ORF) finder tool⁴ were used. Comparative genome alignment with corresponding reference plasmids was performed using Mauve v.2.3.1 (Darling et al., 2010). Figures were generated from sequence data using BRIG v.0.95 (Alikhan et al., 2011) and clinker v0.0.23 (Gilchrist and Chooi, 2021).

Phylogenetic analysis

In total, four different datasets were subjected to phylogenetic analysis. Two of them were local phylogenetic trees including only isolates from our collection: the first one comprised all detected *mcr*-carrying *E. coli* isolates and the second one showed the phylogeny of *Enterobacter* spp. genomes. The third tree was global and comprised genomes of 449 *E. coli* ST744 isolates that were available at Enterobase in April 2021⁵ along with 10 ST744 isolates from our collection. These three trees were generated based on a core-genome determined employing a Roary pipeline v3.12.0 (Page et al., 2015) and aligned with MAFFT v7.313 (Katoh and Standley, 2013). Trees were inferred under the GTR + CAT model using FastTree v2.1.11 (Price et al., 2010) compiled with double precision arithmetic.

Remaining detailed tree topology was constructed based on a pipeline described in a previous study (Forde et al., 2022) using Python scripts that are available on GitHub.⁶ Based on the *E. coli* ST744 global tree, 38 Illumina SRA archives belonging to isolates that were closely related to 10 ST744 isolates from our collection were gathered from the GenBank database in May 2021. Raw sequencing reads of those 38 isolates along with another 10 from our collection were subjected to quality trimming *via* Trimmomatic tool v0.36 (Bolger et al., 2014) and consequently mapped to *E. coli* str. K-12 substr. MG1655 reference genome (GenBank accession U00096.3) using Bowtie2 v2.3.4.2 (Langmead and Salzberg, 2012). Single nucleotide polymorphisms (SNPs) were detected in individual isolates by VarScan v2.4.4 (Koboldt et al., 2012) using the following parameters: minimum read depth of 8; minimum base quality of 20; variant allele frequency ≥ 0.80 . Problematic sites were then removed based on the following rules: occurred in phage regions as detected by PHASTER (Arndt et al., 2016); occurred in repetitive/homologous genomic regions; more than 5 isolates at a particular site showed prevalent base frequency below 80% or/and read depth below 8. The resulting alignment file was then subjected to maximum-likelihood analysis using RAXML v8.2.11 (Stamatakis, 2014) under the GTR + CAT model of nucleotide substitution with 500 rapid bootstrap replicates using sample SRR9990292 as an outgroup. Tree topologies

1 <https://cge.cbs.dtu.dk/>

2 <https://jgi.doe.gov/data-and-tools/software-tools/bbtools/bb-tools-user-guide/>

3 www.ncbi.nlm.nih.gov/BLAST

4 www.bioinformatics.org/sms/

5 <http://enterobase.warwick.ac.uk/>

6 <https://github.com/matejmedvecky/anthraxdiversityscripts>

TABLE 1 The characteristics of sequenced *mcr*-encoding plasmids.

Plasmid name	Organism (ST) ¹	Plasmid-carrying <i>mcr</i> ²	<i>mcr</i>	Other ARGs in <i>mcr</i> plasmid ³	WGS platform	Accession no.
pMCR1-40331	<i>K. pneumoniae</i> (ST290)	IncX4 (33,303)	<i>mcr-1.1</i>	–	Illumina	OP428973
pMCR1-42913	<i>E. coli</i> (ST448)	IncX4 (33,304)	<i>mcr-1.1</i>	–	Illumina	OP428974
pMCR1-44158	<i>E. coli</i> (ST156)	IncX4 (33,303)	<i>mcr-1.2</i>	–	Illumina	OP428975
pMCR1-44653	<i>E. coli</i> (ST1196)	IncX4 (33,303)	<i>mcr-1.1</i>	–	Illumina	OP428976
pMCR1-45082	<i>E. coli</i> (ST744)	IncX4 (34,068)	<i>mcr-1.1</i>	–	Illumina	OP428977
pMCR1-46049	<i>K. pneumoniae</i> (ST147)	IncX4 (33,303)	<i>mcr-1.1</i>	–	Illumina	OP428978
pMCR1-53288	<i>E. coli</i> (ST538)	IncI2 (60,733)	<i>mcr-1.1</i>	–	MinION	OP434482
pMCR1-43934	<i>E. coli</i> (ST8186)	IncHI2/ST4 (225,732)	<i>mcr-1.1</i>	<i>aph(6)-Ia</i> , <i>aph(3)-Ib</i> , <i>catA1</i> , <i>tet(A)</i>	MinION	OP950834
pMCR1-51133	<i>E. coli</i> (ST117)	IncHI2/ST4 (237,743)	<i>mcr-1.1</i>	<i>aadA1</i> , <i>aadA2b</i> , <i>bla_{TEM-1B}</i> , <i>catA1</i> , <i>cmlA1</i> , <i>qacE</i> , <i>sul1</i> , <i>tet(A)</i>	MinION	OP950835
pMCR1-59496	<i>K. pneumoniae</i> (ST726)	IncHI2/ST3 (254,909)	<i>mcr-1.1</i>	<i>aac(3)-IV</i> , <i>aadA1</i> , <i>aadA2b</i> , <i>aph(3')-Ia</i> , <i>aph(4)-Ia</i> , <i>bla_{CTX-M-14}</i> , <i>cmlA1</i> , <i>floR</i> , <i>fosA3</i> , <i>mph(A)</i> , <i>sul2</i> , <i>sul3</i>	MinION	OP950836
pMCR9-16539	<i>E. kobei</i> (ST591)	IncHI2/ST1 (285,283)	<i>mcr-9.1</i>	<i>aadA2b</i> , <i>aph(3')-Ib</i> , <i>aph(6)-Ia</i> , <i>bla_{TEM-1B}</i> , <i>bla_{SHV-12}</i> , <i>catA2</i> , <i>dfrA19</i> , <i>qacE</i> , <i>qnrA1</i> , <i>sul1</i> , <i>tet(D)</i>	MinION	OP950838
pMCR9-17620	<i>E. hormaechei</i> (ST91)	IncHI2/ST17 (276,870)	<i>mcr-9.1</i>	<i>aadA2b</i> , <i>ant(2'')-Ia</i> , <i>bla_{CTX-M-9}</i> , <i>catA1</i> , <i>dfrA16</i> , <i>qacE</i> , <i>qnrA1</i> , <i>sul1</i>	MinION	OP950833
pMCR9-57185	<i>C. freundii</i> (ST18)	IncHI2/ST1 (330,692)	<i>mcr-9.1</i>	<i>aac(6')-IIc</i> , <i>bla_{TEM-1B}</i> , <i>bla_{DHA-1}</i> , <i>catA2</i> , <i>ere(A)</i> , <i>qacE</i> , <i>qnrB4</i> , <i>sul1</i> , <i>tet(D)</i>	MinION	OP950837
pMCR4-26153	<i>E. kobei</i> (ST54)	ColE10 (12,808)	<i>mcr-4.2</i>	–	MinION	OP428979

¹ST, sequence type.

²Plasmid carrying *mcr* include the information on plasmid incompatibility group (Inc), ST (if available) and plasmid size in bp.

³ARGs, antibiotic resistance genes.

were visualized via iTOL v6.3 (Letunic and Bork, 2021) and edited using Inkscape v1.1.7. The SNP distance matrix was generated by snp-dists 0.7.0 software.⁸

Nucleotide sequence accession numbers

Genome assemblies, SRA archives and annotated plasmid sequences (Table 1) were deposited in the NCBI under BioProject with accession number PRJNA772899.

Statistical analysis

The statistical significance of the increasing prevalence of *mcr* genes between 2018 and 2020 was calculated using Fisher's exact test (two tailed) using a significance level $\alpha = 0.05$.

Results

mcr-positive *Enterobacterales* isolates

From all 1932 examined colistin-resistant isolates, 73 (3.8%) were identified to carry *mcr* genes (Supplementary Table S1). Most (65/73) isolates were detected during the prospective years, including eight isolates in 2018 (3%, $n = 274$), 27 isolates in 2019 (4%, $n = 634$) and 30

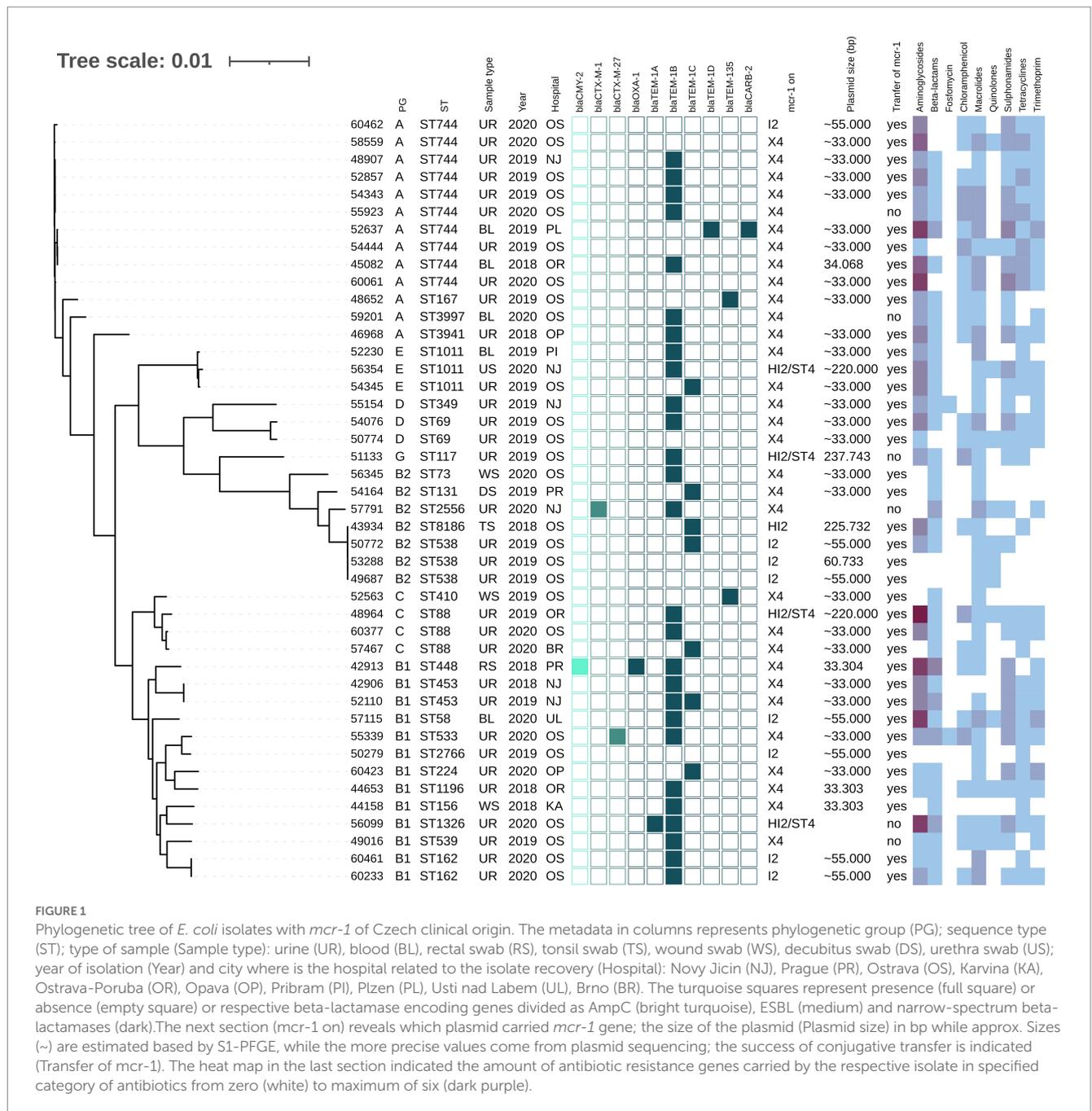
isolates in 2020 (9%, $n = 342$). The difference in increasing prevalence of *mcr* genes between 2018 and 2020 was statistically significant ($p < 0.007$). From the retrospective analysis using a collection of isolates at the National Reference Laboratory for Antibiotics, seven isolates (1%, $n = 682$) carrying *mcr* genes were found, including seven *Enterobacter* spp. from 2010 ($n = 1$), 2012 ($n = 3$), 2013 ($n = 1$), 2014 ($n = 2$) and one isolate of *K. pneumoniae* ($n = 1$) from 2017. Isolates carrying *mcr-1* were identified as *E. coli* ($n = 44$) and *K. pneumoniae* ($n = 4$), while the remaining 25 isolates carried the *mcr-9.1* allele. Three of the *mcr-9.1*-carrying isolates were also positive for *mcr-4.2/mcr-4.3*. The isolates carrying *mcr-9.1* were identified as *Citrobacter freundii* ($n = 1$), *Enterobacter asburiae* ($n = 13$), *Enterobacter kobei* ($n = 6$), *Enterobacter cloacae* ($n = 3$), *Enterobacter roggenkampii* ($n = 1$) and *Enterobacter hormaechei* ($n = 1$). Plasmid-mediated colistin resistance genes were the most common among *E. coli* as 19% (44/231) colistin-resistant isolates carried *mcr-1*, while the occurrence in other species was rare (4.4% in *Enterobacter* spp., 0.4% in *K. pneumoniae*).

Colistin-resistant isolates carrying *mcr-1* (48/73, 66%) showed phenotypic resistance to beta-lactam antibiotics including ampicillin (46/48, 96%), ampicillin/sulbactam (43/48, 90%), cefoxitin (9/48, 19%), piperacillin/tazobactam (9/48, 19%), cefotaxime (5/48, 10%), ceftazidime (5/48, 10%), cefepime (4/48, 8%) and ceftolozane/tazobactam (2/48, 4%). Resistance to other antimicrobials including cotrimoxazole (34/48, 71%), ciprofloxacin (34/48, 71%), trimethoprim (34/48, 71%), gentamicin (11/48, 23%), tobramycin (10/48, 21%) and amikacin (1/48, 2%) was found.

The majority of colistin-resistant isolates carrying *mcr-9* were resistant to cefoxitin (25/25, 100%), ampicillin (23/25, 92%), ampicillin/sulbactam (23/25, 92%) and cefotaxime (12/25, 48%). Furthermore, they showed resistance to ceftazidime (9/25, 36%), cotrimoxazole (6/25, 24%), ciprofloxacin (5/25, 20%), tobramycin (5/25, 20%), trimethoprim (5/25, 20%), piperacillin/tazobactam (7/25,

⁷ <https://inkscape.org/cs/>

⁸ <https://github.com/tseemann/snp-dists>



28%), gentamicin (4/25, 16%), cefepime (3/25, 12%), amikacin (2/25, 8%), ceftolozane/tazobactam (2/25, 8%) and meropenem (2/25, 8%).

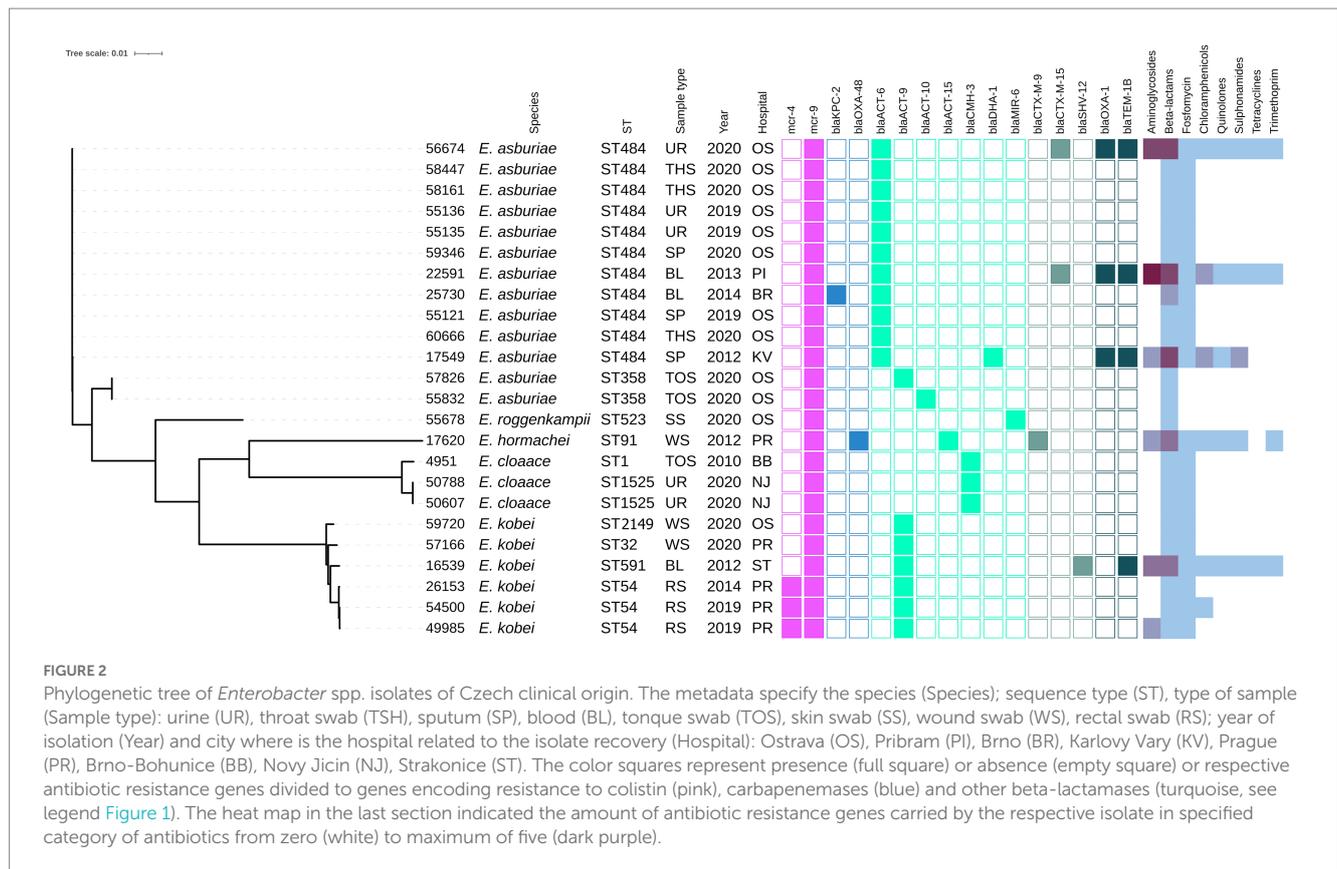
Nine isolates, including *E. coli* ($n=4$), *Enterobacter* spp. ($n=3$), *Citrobacter freundii* ($n=1$) and *K. pneumoniae* ($n=1$) with resistance to seven or more different antibiotics were simultaneously positive for ESBL production. AmpC beta-lactamase was detected in six *Enterobacter* spp. isolates and one *E. coli* isolate.

Analysis of WGS results

The *mcr-1*-positive *E. coli* isolates belonged to 26 various STs of which *E. coli* ST744 was the most common (10/44). Fifteen *E. coli* isolates were assigned to ST88 ($n=3$), ST538 ($n=3$), ST1011 ($n=3$),

ST69 ($n=2$), ST162 ($n=2$), and ST453 ($n=2$), while the remaining 19 isolates belonged to unique STs (Supplementary Table S1 and Figure 1). Four *K. pneumoniae* isolates carrying *mcr-1* were assigned to four different STs (ST147, ST231, ST290, and ST726) and one *C. freundii* isolate with *mcr-9* belonged to ST18. *Enterobacter* spp. with *mcr-9* belonged predominantly to *E. asburiae* of two different STs, including ST484 ($n=11$) and ST358 (2) (Figure 2). Six isolates of *E. kobei* belonged to ST32 ($n=1$), ST2149 ($n=1$), ST591 ($n=1$), and ST54 ($n=3$). All *E. kobei* ST54 isolates originated from patients in a single hospital and carried *mcr-4* apart from *mcr-9*. Two remaining isolates were identified as *E. cloacae* ST1525 and were obtained from one patient.

Most *mcr-1*-positive isolates carried genes (Supplementary Table S1) conferring resistance to aminoglycosides



(36/48), macrolides (42/48), sulphonamides (36/48), tetracycline (33/48) and trimethoprim (31/48). Additionally, 44 *mcr-1*-positive isolates harbored genes for resistance to narrow-spectrum beta-lactams, including *bla*_{TEM-1B} ($n=27$), *bla*_{TEM-135} ($n=4$) and *bla*_{TEM-32} ($n=3$). In two isolates, AmpC beta-lactamase genes *bla*_{CMY-2} or *bla*_{DHA-1} were detected, while in three isolates, the ESBL genes *bla*_{CTX-M-1} ($n=1$), *bla*_{CTX-M-27} ($n=2$) were found. All four *mcr-1*-positive *K. pneumoniae* isolates carried *fosA*, *oqxA*, *oqxB* and *bla*_{SHV} genes.

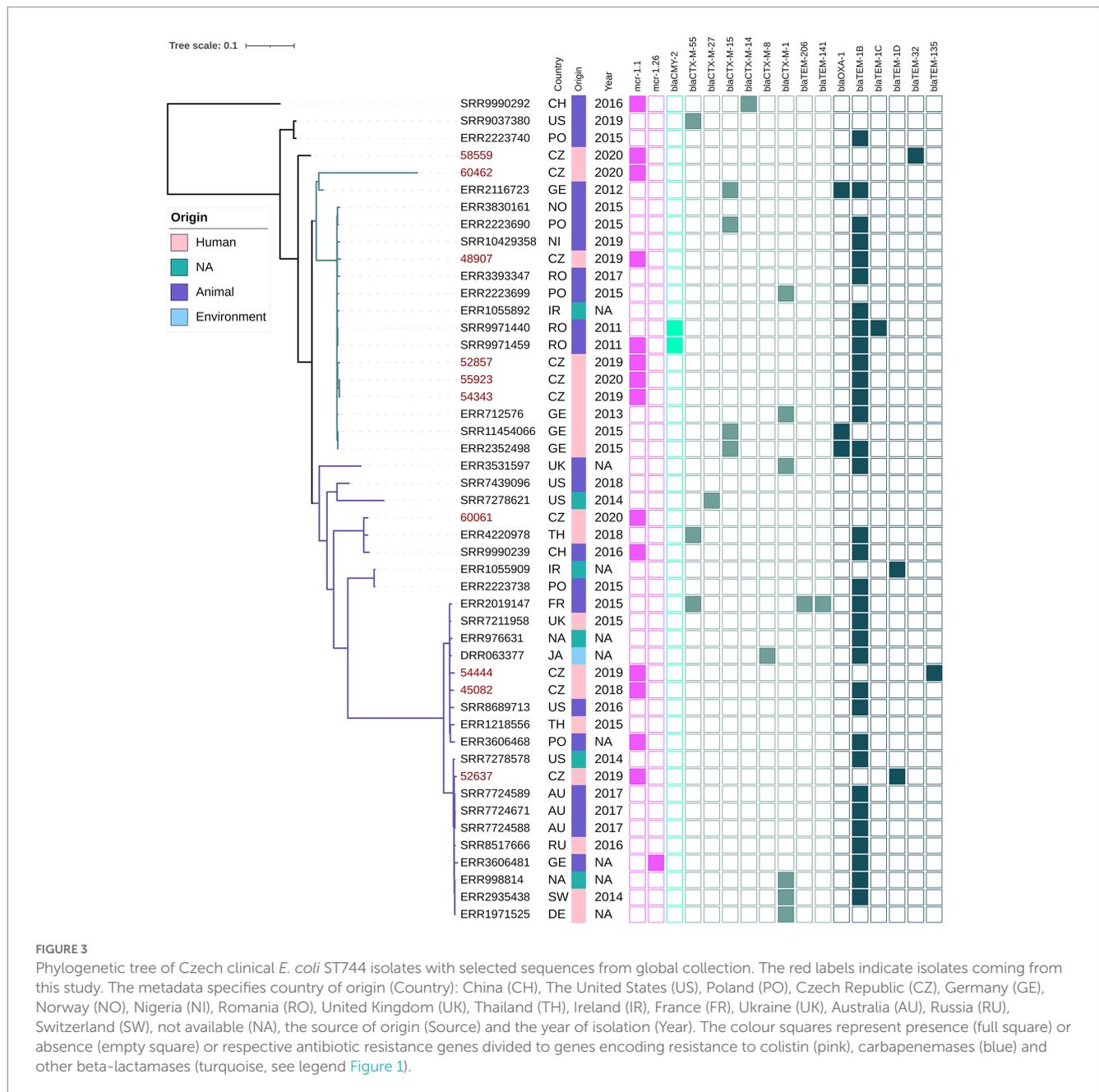
On the other hand, the majority of *mcr-9*-positive isolates contained resistance genes to beta-lactams ($n=25$) and fosfomycin ($n=22$). Specifically, the *E. asburiae* ST484 isolates carried the *bla*_{ACT-6} gene, encoding the intrinsic AmpC beta-lactamase, while the *E. asburiae* ST358 isolates carried *bla*_{ACT-9} or *bla*_{ACT-10} variants (Figure 2). The two *E. cloacae* ST1525 isolates harbored only the *bla*_{CMH-3} gene, encoding the chromosomal AmpC. The ST54 *E. kobei* isolates carried the *bla*_{ACT-9} and *fosA* genes. Moreover, ESBL (*bla*_{CTX-M-9}, *bla*_{CTX-M-15}) and AmpC beta-lactamase (*bla*_{DHA-1}, *bla*_{CMY-117}) were identified in three and two *mcr-9*-positive isolates, respectively. One *E. hormaechei* ST91 isolate carried carbapenemase gene *bla*_{OXA-48} on IncL plasmid, while the single *Citrobacter freundii* ST18 isolate carried five beta-lactamase genes, including carbapenemase-encoding gene *bla*_{KPC-2} carried by IncR plasmid.

Chromosomal mutations to fluoroquinolones (*acrR*), cephalosporins (*ompK36*) and carbapenems (*ompK37*) were identified in four *K. pneumoniae* isolates (Supplementary Table S1). In contrast with the susceptibility testing, three of these *K. pneumoniae* isolates were resistant to fluoroquinolones and only one to cephalosporins. Twenty-five *E. coli* isolates carried at least one of four mutation variants, including *gyrA*, *gyrB*, *parC*, and *parE* for resistance to quinolones, and

13 *E. coli* isolates carried quinolone-resistance genes *qnrB* ($n=5$) or *qnrS* ($n=8$) (Supplementary Table S1) while MIC profiles showed quinolone resistance in 31 isolates. Nine different types of mutations in *pmrA/pmrB* genes associated with colistin resistance were also found in 11 *E. coli* isolates (Supplementary Table S1).

Phylogenetic analysis of *Escherichia coli* ST744

Phylogenetic analysis of ST744 isolates from our collection ($n=10$) along with other 38 closely related genomes from public resources showed the formation of two major clades; four isolates were considered as outliers (Figure 3). The first major clade (samples 60462-ERR2352498, green branch) comprised 17 mostly European isolates from animals and humans, and was further divided into two subclades. The second dominant clade (samples ERR3531597 – ERR1971525, violet branch) was composed of 27 cosmopolitan isolates originating from various sources, and was divided into several smaller subclades. Isolates belonging to different major clades showed a variable number of pairwise SNP differences against each other, ranging from 600 up to 3,000. In both major clades, there were apparent clusters of isolates from humans and animals, exhibiting a few dozen of SNPs from each other (Supplementary Table S2). Isolates from our collection were scattered across the tree, five of them belonged to the first clade, four to the second clade, while one sample was an outlier. Our clinical samples 48907, 52857, 55923 and 54343, belonging to the first clade, showed 46–58 SNP differences from three clinical samples from Germany, and 39–49 SNP differences from the



two Romanian (RO) isolates from poultry that carried *bla*_{CMY-2}. One of the RO isolates also carried the *mcr-1.1* gene that was borne by all isolates from our Czech clinical collection. Those RO isolates were also closely related to three clinical isolates from Germany (exhibited <20 SNP differences from each other). Isolate 60061 from the Czech collection clustered with a clinical isolate from Thailand (110 SNPs) and a Chinese isolate from a pig (128 SNPs). Notably, the Swiss isolate also carried *mcr-1.1*. Our isolates 45082 and 54444 were related to another clinical isolate from the United Kingdom (66 and 73 SNPs) and also to an environmental isolate from a river in Japan (73, and 80 SNPs, respectively). Isolate 52637 from our collection showed the least SNP counts against three Australian isolates from gulls (36–37 SNPs) and three clinical isolates, one from Switzerland (36 SNPs), and another from Germany (39 SNPs), and Russia (42 SNPs).

Structure of *mcr-1*-carrying plasmids

The *mcr-1* gene was located predominantly on 33 kb IncX4 plasmids (34/48). Six complete plasmids from *E. coli* and *K. pneumoniae* obtained by long-read sequencing (Table 1) showed a high level of nucleotide similarity (>99.9%) to each other as well as to plasmids from raw meat from Czech retails (Zelendova et al., 2021; Supplementary Figure S1). The *mcr-1* gene was bordered by a hypothetical protein and a PAP2 transmembrane protein, which is the typical genetic surrounding for *mcr-1* gene within IncX4 plasmids (Zelendova et al., 2021).

The *mcr-1* was also carried by ~60 kb IncI2 plasmids ($n=8$). Plasmid pMCR1-53288 originating from *E. coli* ST538 from urine obtained by MinION sequencing shared high sequence similarity

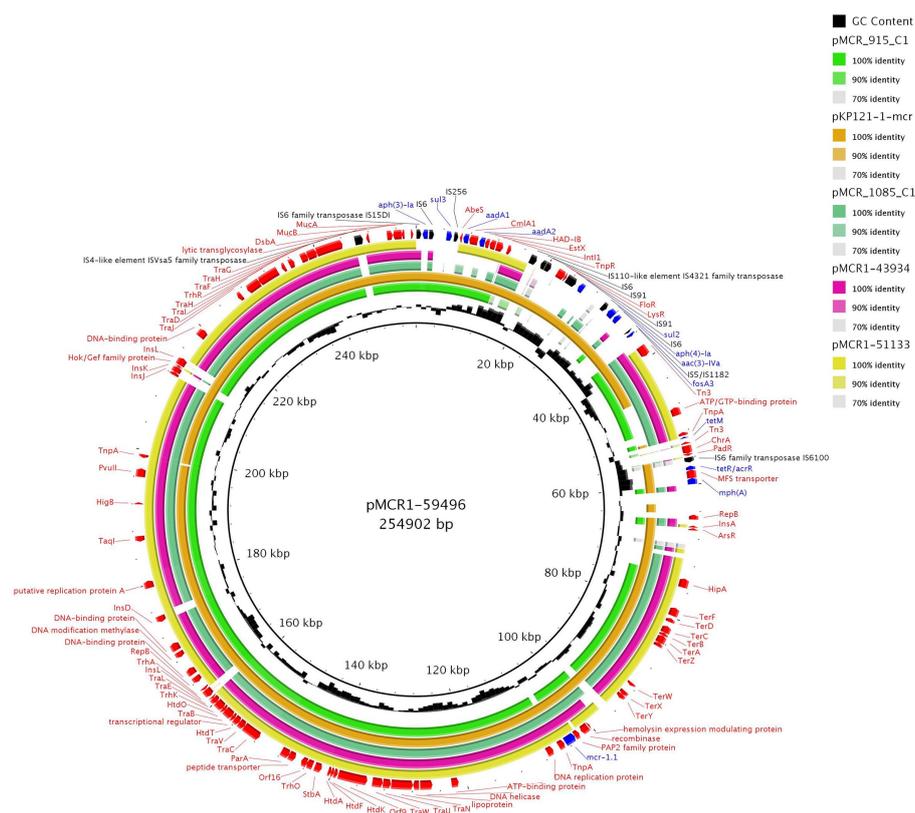


FIGURE 4

BRIG comparison of *mcr-1.1*-encoding IncHI2/ST4 plasmids. The plasmid pMCR1-59496 from *K. pneumoniae* ST276 identified in our study from urine sample (OP950836) was used as a reference. Two other plasmids originated from our collection, including pMCR1-43934 from *E. coli* ST8186 from tonsil swab sample and pMCR1-51133 from *E. coli* ST117 from a urine sample. The sequence alignment includes pMCR_915_C1 (MT929284.1) and pMCR_1085_C1 (MT929286.1) from *E. coli* recovered from raw turkey meat imported to the Czech Republic from Poland and one of plasmid pKP121-1-mcr (CP031850.1) from *K. pneumoniae* ST2570 from human blood in China.

(>98%) with several plasmids available in the GenBank database, including pMCR_1884_C3 and pMCR_1138_A1 from *C. braakii* and *E. coli* ST162, respectively, isolated from imported meat sold in Czech retailers (Zelendova et al., 2021; Supplementary Figure S2). The *mcr-1* region was inserted downstream of the *nikB* gene, encoding a DNA topoisomerase III, as observed in other IncI2 *mcr-1*-positive plasmids like pMCR_1884_C3. No other resistance genes were located on IncI2 plasmids.

From our collection, six *Enterobacteriales* isolates were found to harbor IncHI2 plasmids with the *mcr-1* gene. The complete sequence of three *mcr-1*-positive IncHI2 plasmids, pMCR1-59496, pMCR1-43934, and MCR1-51133, was determined using MinION technology. BlastN analysis showed that all sequenced IncHI2 plasmids, ranging from ~225 to ~255 kb in size, belonged to ST4 and were closely related (coverage 80–99%, identity 99%) to each other (Figure 4), as well as to other *mcr-1*-carrying IncHI2 plasmids, like pMCR_915_C1 and pMCR_1085_C1 from *E. coli* recovered from imported meat (Zelendova et al., 2021), and plasmid pKP121-1-mcr (Ruan et al., 2019) of human clinical origin from China. All IncHI2 plasmids contained regions for conjugative transfer (*hdt*, *orf*, *tra* genes) and plasmid maintenance (*par* gene). Additionally, IncHI2 plasmids carried tellurium resistance genes in two clusters, including *terZABCDEF* and *terXYW* (except p56099). In all IncHI2 plasmids, characterized during this study, the *mcr-1* gene was inserted

downstream of the *terY2* gene, as observed in other IncHI2 plasmids like pMCR_1085_C1. Similar to pMCR_1085_C1, the *mcr-1* gene was bounded by an IS*ApI1* element and PAP2 transmembrane protein (Zelendova et al., 2021). All IncHI2 *mcr-1*-positive plasmids exhibited at least one MDR region, which ranged in size from 950 to 36,097 bp (Figure 4).

Structure of *mcr-4*-encoding plasmids

mcr-4 was located on ColE10 plasmids in three *E. kobei* ST54 isolates. Plasmid pMCR4-26153 of size 12,808 bp recovered from a rectal swab of a patient in the Czech Republic was identical (100% coverage, 100% identity) to pIB2020_ColE_MCR (Marchetti et al., 2021) from *E. kobei* ST54 strain from a rectal swab of a 56 years old male patient hospitalized in 2019 in Italy (Supplementary Figure S3).

Structure of *mcr-9.1*-carrying elements

Out of the 25 *mcr-9.1*-positive isolates, eight were characterized by MinION technology. Among the latter isolates, three carried the *mcr-9.1* allele on IncHI2 plasmids (Table 1) while, in the five remaining isolates, the *mcr-9.1* was found on the chromosome.

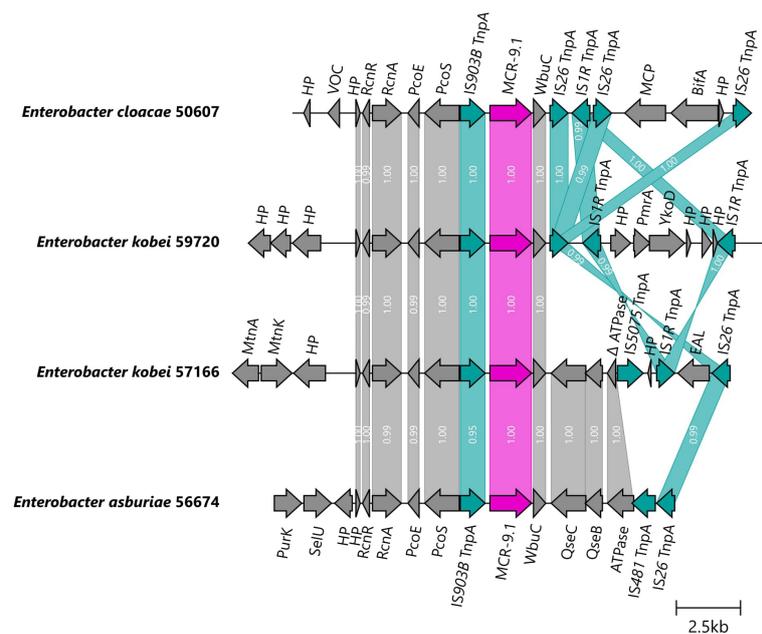


FIGURE 5

Genetic surroundings of *mcr-9.1* gene located on chromosomes in *Enterobacter*. The linearized coding sequences of MCR-9.1 region of four isolates were compared using clinker with identity threshold 90%. The MCR-9.1 (pink) surrounded by mobile genetic elements (turquoise) and other coding sequences (gray) in isolates 50607 and 59720 formed a region corresponding to the *mcr-9* region observed in IncHI2/ST1 plasmids. On the other hand, 57166 and 56674 isolates contained downstream sequence similar to the previously described *mcr-9* environment in *E. asburiae* (AP022628). White numbers in links correspond to the similarity of coding sequences.

Plasmid pMCR9-57185 originated from *C. freundii* ST18 recovered from a rectal swab, while pMCR9-16539 was obtained from *E. kobei* ST591 from blood and pMCR9-17620 came from *E. hormaechei* ST91 recovered from a wound swab.

Following the IncHI2 pDLST scheme, plasmids pMCR9-57185 and pMCR9-16539 were typed as ST1, while pMCR9-17620 was assigned to ST17. All plasmids exhibited closely related sequences (>89% coverage, 99.99% identity) to other *mcr-9.1*-positive IncHI2 plasmids (Supplementary Figure S4), like p49790_MCR from an *E. hormaechei* isolate recovered previously from Czech hospitals (Bitar et al., 2020). Similar to p49790_MCR, the *mcr-9.1* was inserted upstream of the *pcoS* gene (encoding a membrane protein for resistance to copper), in all IncHI2 plasmids like p49790_MCR. Additionally, in plasmids pMCR9-57185 and pMCR9-16539, the *mcr-9.1* gene was bounded by an IS element (upstream) and an ORF (downstream), encoding a cupin fold metalloprotein, followed by IS26. However, in plasmid pMCR9-17620, an IS1 was found downstream of *mcr-9.1*. Furthermore, IncHI2 plasmids contained at least one MDR region, including genes for resistance to aminoglycosides, tetracyclines, trimethoprim, chloramphenicol, sulfonamides, quinolones, and/or macrolides (Table 1). Moreover, IncHI2 plasmids carried tellurium resistance genes (*terZABCDEF*) commonly associated with this plasmid family, and genes conferring arsenic resistance (*arsCBRH*).

The *mcr-9.1* gene was integrated into the chromosomes of four *E. cloacae* complex isolates obtained by long-read assembly. The upstream genetic surroundings were identical in all isolates, consisting of *mcr-9.1*, *IS903B*, *pcoS*, *pcoE*, *rcnA*, and *rcnR* genes, while the downstream sequences differed. In isolates 50607 and 59720, the *mcr-9.1* was followed (downstream) by *wbuC*, IS26 and IS1A forming a

region *rcnR-rcnA-pcoE-pcoS-IS903B-mcr-9.1-wbuC-IS26* identical to the respective region of the plasmid pMCR9-57185. On the other hand, the downstream environment of *mcr-9.1* in isolates 56674 and 57166 consisted of *wbuC*, *qseC*, *qseB* and ATPase ORF similar to the corresponding region in the chromosome of a Japanese human isolate *Enterobacter asburiae* A2563 (AP022628), visualized in Figure 5.

Horizontal transfer of *mcr* gene

Resistance to colistin associated with *mcr-1* was transferred to recipient *E. coli* laboratory strains *via* conjugation in the majority of *E. coli* isolates (38/44, 86%) and in all isolates of *K. pneumoniae* (4/4, 100%). The most frequently transferred plasmid harboring *mcr-1* included 33 kb plasmid IncX4 (31/34, 91%), followed by 55 kb IncI2 (8/8, 100%) and 220 kb IncHI2 (4/6, 67%) plasmids. IncHI2 plasmid with *mcr-9* was transferred *via* conjugation in one *E. asburiae* isolate. Also, ColE10 plasmid carrying *mcr-4* was not transferred *via* conjugation, since this plasmid family does not contain any transfer region.

Discussion

Within this study, we performed a surveillance of *mcr*-encoding genes among colistin-resistant *Enterobacterales* collected from Czech hospitals between 2009 and 2020. Our findings indicated a low prevalence of *mcr* genes among colistin-resistant isolates, with a slightly increasing prevalence during the study period. However, the prevalence of *mcr*-positive isolates may be overestimated since our

collection was composed only of colistin-resistant isolates and did not include the bacterial population susceptible to colistin. Moreover, another study limitation is the fact that isolates from retrospective sampling from the period 2009–2017 were obtained during various surveillance programmes at the National Institute of Public Health. As these programmes were focused mainly on *Klebsiella* sp., invasive *E. coli* or they were targeting MDR strains, the data of *mcr* prevalence from this period needs to be interpreted with caution.

Other studies from Europe have reported a low prevalence of colistin-resistant isolates and of *mcr*-positive *Enterobacterales*. The Czech study by Tkadlec et al. (2021) found *E. coli* with *mcr-1* gene in only 0.21% (4/1922) of fecal samples from hospitalized patients. A study from Switzerland reported that the fecal carriage rate of colistin-resistant (MIC value >2 mg/L) *Enterobacterales* was 1.5% for healthy people and 3.8% for primary care patients (Zurfluh et al., 2017). Additionally, in Finland, only one *mcr-1*-positive *E. coli* was characterized from fecal samples collected from 176 healthy volunteers (Gröndahl-Yli-Hannuksela et al., 2018). In Spain, the overall prevalence of colistin resistance in clinical isolates of *Enterobacterales* was 0.67% and *mcr-1* was detected only in *E. coli* (0.15%) (Prim et al., 2017). Similar prevalence levels were observed for Romagna, Northern Italy, where the prevalence of colistin-resistant isolates among human *Enterobacterales* was 0.5% and the *mcr-1* gene was found in 0.14% *E. coli* isolates (Del Bianco et al., 2018). On the other hand, higher percentages of *mcr*-carrying isolates have been reported in some areas outside Europe. Giani et al. (2018) reported a high proportion (38.8%) of *mcr-1* carriers among healthy children (129/337) from Bolivia. Furthermore, in Chinese hospitals across 24 provincial capital cities and municipalities, human carriage of *mcr-1*-positive *E. coli* was identified in 644 (14.3%) of 4,498 samples in 2016 (Wang Y. et al., 2020). However, different methodological approaches, study designs and sample types (e.g., selective cultivation on colistin-supplemented media, targeting isolates despite their susceptibility profiles, PCR detection of *mcr* genes in either total enterobacterial gut microbiota or directly in a clinical sample) significantly limit the comparison of prevalence data between the studies. Moreover, the discrepancy in the prevalence of *mcr* carriers between studies and geographical regions underlines the other factors, like antibiotic use and stewardship protocols, contributing to the emergence and spread of colistin-resistant isolates.

In this study, the majority of isolates carried *mcr-1* followed by *mcr-9* and *mcr-4* variants. Most *mcr-1* carriers were *E. coli* and the gene was present in 23% of all resistant isolates of this species, which is in agreement with findings of previous study (Zelendova et al., 2021). Most of the *mcr-9.1*-carrying isolates in our study belonged to *Enterobacter* spp. Previous studies have shown that the *mcr-9* gene is commonly associated with isolates belonging to the *Salmonella* and *Enterobacter* genus (Bitar et al., 2020; Liao et al., 2022; Zhang et al., 2022). Interestingly, the low resistance levels to colistin of MCR-9-producing *Enterobacter* isolates have been reported (Bitar et al., 2020). This observation may explain the unnoticed spread of those isolates in Czech hospitals. Remarkably, 96% of isolates (70/73) carried AmpC/ESBL or carbapenemases, raising the concern that the spread of *mcr*-carrying isolates might also be related to the use of other antimicrobial agents, including clinically important beta-lactams.

MLST revealed the presence of *mcr* genes in various STs of *E. coli*, *K. pneumoniae*, and *Enterobacter* sp., highlighting the significant impact of horizontal gene transfer in the spread of colistin resistance determinants via plasmids. Additionally, phylogenetic analysis

uncovered the association of *mcr* genes with specific clones, like *E. kobei* ST54, which has been previously reported to produce MCR-4.3 from clinical samples recovered in Italy (Marchetti et al., 2021). Of note, these observations underline the important role of traveling across borders, that has contributed to the spread of MDR bacteria.

Finally, analysis of *mcr*-carrying plasmid sequences showed the presence of *mcr-1*, mainly on IncX4 replicons, but also on IncI2 and IncHI2 plasmids. These findings are in agreement with the previously published data, showing the emergence of *mcr* genes on the specific Inc groups of plasmids that were characterized from *Enterobacterales* recovered from different sources, including animals, food and humans (Xavier et al., 2016; Zurfluh et al., 2017; El Garch et al., 2018; Bitar et al., 2020; Li et al., 2020; Marchetti et al., 2021; Zelendova et al., 2021). Furthermore, our experiments demonstrated a high efficiency of conjugative transfer of *mcr-1*-carrying IncX4 plasmids. Also, the conjugative transfer of IncHI2 plasmids carrying *mcr-1* or *mcr-9* was confirmed. Thus, the horizontal transfer of plasmid-mediated *mcr* genes represents an important risk factor for public health since colistin is considered as one of the last-resort antibiotics for the treatment of serious infections in human medicine. Therefore, studying the spread of MDR pathogens is vital for analysis of transmission pathways and risk factors for public health.

The prospective epidemiological survey performed in this study brought the first information on the plasmid-mediated dissemination in the Czech Republic and showed that a surveillance system is essential to monitor the diffusion of plasmid mediated colistin resistance.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/genbank/>, PRJNA772899.

Ethics statement

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Author contributions

MZ performed laboratory work, data analysis, and prepared the manuscript. CP performed data analysis and prepared the manuscript. PS performed laboratory work and helped with the manuscript preparation. MM performed bioinformatic analyses of whole-genome sequencing data. JP conducted MinION sequencing and helped with plasmid analysis. KN contributed on figure preparation. VJ, KP, and HZ provided the samples. IJ performed whole-genome sequencing. MD supervised the project, performed data analysis, and revised the manuscript. All authors discussed the results. Members of the

surveillance network provided the clinical isolates and metadata obtained during the standard microbiological testing in their laboratories.

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Funding

This project was funded by projects of Czech Health Research Council NV18-09-0060 and NU20J-09-0040, the Internal Grant Agency 205/2022/FVHE and partially Ministry of Health, Czech Republic – conceptual development of research organization (FNBr, 65269705).

Acknowledgments

We thank Dana Cervinkova, Iva Sukkar, and Katarina Stredanska for their assistance in the laboratory and to Adam Valcek for his help with data analysis.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2023.1147846/full#supplementary-material>

References

- Aghapour, Z., Gholizadeh, P., Ganbarov, K., bialvaei, A. Z., Mahmood, S. S., Tanomand, A., et al. (2019). Molecular mechanisms related to colistin resistance in Enterobacteriaceae. *Infect. Drug Resist.* 12, 965–975. doi: 10.2147/IDR.S199844
- Alikhan, N. F., Petty, N. K., Ben Zakour, N. L., and Beatson, S. A. (2011). BLAST Ring Image Generator (BRIG): simple prokaryote genome comparisons. *BMC Genomics* 12, 1–10. doi: 10.1186/1471-2164-12-402

- Arndt, D., Grant, J. R., Marcu, A., Sajed, T., Pon, A., Liang, Y., et al. (2016). PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic Acids Res.* 44, W16–W21. doi: 10.1093/nar/gkw387
- Bankevich, A., Nurk, S., Antipov, D., Gurevich, A. A., Dvorkin, M., Kulikov, A. S., et al. (2012). SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *J. Comput. Biol.* 19, 455–477. doi: 10.1089/cmb.2012.0021
- Bauer, A. P., Dieckmann, S. M., Ludwig, W., and Schleifer, K.-H. (2007). Rapid identification of *Escherichia coli* safety and laboratory strain lineages based on multiplex-PCR. *FEMS Microbiol. Lett.* 269, 36–40. doi: 10.1111/j.1574-6968.2006.00594.x
- Bitar, I., Medvecký, M., Gelbicova, T., Jakubu, V., Hrabak, J., Zemlickova, H., et al. (2019). Complete Nucleotide Sequences of *mcr-4.3*-Carrying Plasmids in *Acinetobacter baumannii* Sequence Type 345 of Human and Food Origin from the Czech Republic, the First Case in Europe. *Antimicrob. Agents Chemother.* 63. doi: 10.1128/aac.01166-19
- Bitar, I., Papagiannitsis, C. C., Kraftova, L., Chudejova, K., Mattioni Marchetti, V., and Hrabak, J. (2020). Detection of five *mcr-9*-carrying *Enterobacteriales* isolates in four czech hospitals. *mSphere* 5:e01008-20. doi: 10.1128/mSphere.01008-20
- Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170
- Borowiak, M., Hammerl, J. A., Deneke, C., Fischer, J., Szabo, I., and Malorny, B. (2019). Characterization of *mcr-5*-harboring *Salmonella enterica* subsp. *enterica* serovar typhimurium isolates from animal and food origin in germany. *Antimicrob. Agents Chemother.* 63:e00063-19. doi: 10.1128/aac.00063-19
- Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K. L., and Threlfall, E. J. (2005). Identification of plasmids by PCR-based replicon typing. *J. Microbiol. Methods* 63, 219–228. doi: 10.1016/j.mimet.2005.03.018
- Carattoli, A., Villa, L., Feudi, C., Curcio, L., Orsini, S., Luppi, A., et al. (2017). Novel plasmid-mediated colistin resistance *mcr-4* gene in *Salmonella* and *Escherichia coli*, Italy 2013, Spain and Belgium, 2015 to 2016. *Eur. Secur.* 22:30589. doi: 10.2807/1560-7917.ES.2017.22.31.30589
- Carattoli, A., Zankari, E., García-Fernández, A., Voldby Larsen, M., Lund, O., Villa, L., et al. (2014). In silico detection and typing of plasmids using plasmidfinder and plasmid multilocus sequence typing. *Antimicrob. Agents Chemother.* 58, 3895–3903. doi: 10.1128/AAC.02412-14
- Centers for Disease Control and Prevention (2004). *Standardized molecular subtyping of foodborne bacterial pathogens by pulse-field gel electrophoresis*. Centers for Disease Control and Prevention, Atlanta, GA.
- Dalmolin, V. T., de Lima-Morales, D., and Barth, L. A. (2018). Plasmid-mediated colistin resistance: what do we know? *J. Infect.* 1, 16–22. doi: 10.29245/2689-9981/2018/2.1109
- Darling, A. E., Mau, B., and Perna, N. T. (2010). progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. *PLoS One* 5:e11147. doi: 10.1371/journal.pone.0011147
- Del Bianco, F., Morotti, M., Pedna, M. F., Farabegoli, P., and Sambri, V. (2018). Microbiological surveillance of plasmid mediated colistin resistance in human *Enterobacteriaceae* isolates in Romagna (Northern Italy): August 2016–July 2017. *Int. J. Infect. Dis.* 69, 96–98. doi: 10.1016/j.ijid.2018.02.006
- Doumith, M., Godbole, G., Ashton, P., Larkin, L., Dallman, T., Day, M., et al. (2016). Detection of the plasmid-mediated *mcr-1* gene conferring colistin resistance in human and food isolates of *Salmonella enterica* and *Escherichia coli* in England and Wales. *J. Antimicrob. Chemother.* 71, 2300–2305. doi: 10.1093/jac/dkw093
- ECDC, European Centre for Disease Prevention and Control (2019). ECDC Technical Report, Expert consensus protocol on colistin resistance detection and characterisation for the survey of carbapenem- and/or colistin-resistant *Enterobacteriaceae*. Available at: <https://www.ecdc.europa.eu/sites/default/files/documents/expert-consensus-protocol-colistin-resistance.pdf>
- El Garch, F., de Jong, A., Bertrand, X., Hocquet, D., and Sauguet, M. (2018). *mcr-1*-like detection in commensal *Escherichia coli* and *Salmonella* spp. from food-producing animals at slaughter in Europe. *Vet. Microbiol.* 213, 42–46. doi: 10.1016/j.vetmic.2017.11.014
- El-Sayed Ahmed, M. A. E.-G., Zhong, L.-L., Shen, C., Yang, Y., Doi, Y., and Tian, G.-B. (2020). Colistin and its role in the Era of antibiotic resistance: an extended review (2000–2019). *Emerg. Microbes Infect.* 9, 868–885. doi: 10.1080/22221751.2020.1754133
- European Committee on Antimicrobial Susceptibility Testing. (2017). Breakpoint table for interpretation of MICs and zone diameters. Version 2.0. Available at: <http://www.eucast.org>.
- Forde, T. L., Dennis, T. P. W., Aminu, O. R., Harvey, W. T., Hassim, A., Kiwelu, I., et al. (2022). Population genomics of *Bacillus anthracis* from an anthrax hyperendemic area reveals transmission processes across spatial scales and unexpected within-host diversity. *Microb. Genomics* 8:759. doi: 10.1099/mgen.0.000759
- Giani, T., Sennati, S., Antonelli, A., Di Pilato, V., di Maggio, T., Mantella, A., et al. (2018). High prevalence of carriage of *mcr-1*-positive enteric bacteria among healthy children from rural communities in the Chaco region, Bolivia, September to October 2016. *Eur. Secur.* 23:1800115. doi: 10.2807/1560-7917.ES.2018.23.45.1800115
- Gilchrist, C. L., and Chooi, Y. H. (2021). Clinker & clustermap. js: Automatic generation of gene cluster comparison figures. *Bioinformatics* 37, 2473–2475. doi: 10.1093/bioinformatics/btab007
- Gröndahl-Yli-Hannuksela, K., Lönnqvist, E., Kallonen, T., Lindholm, L., Jalava, J., Rantakokko-Jalava, K., et al. (2018). The first human report of mobile colistin resistance gene, *mcr-1*, in Finland. *APMIS* 126, 413–417. doi: 10.1111/apm.12834
- Hamel, M., Rolain, J.-M., and Baron, S. A. (2021). The history of colistin resistance mechanisms in bacteria: progress and challenges. *Microorganisms* 9:442. doi: 10.3390/microorganisms9020442
- Javed, H., Saleem, S., Zafar, A., Ghafoor, A., Shahzad, A. B., Ejaz, H., et al. (2020). Emergence of plasmid-mediated *mcr* genes from Gram-negative bacteria at the human-animal interface. *Gut Pathogens* 12:54. doi: 10.1186/s13099-020-00392-3
- Katip, W., Yoodce, J., Uitrakul, S., and Oberdorfer, P. (2021). Efficacy of loading dose colistin versus carbapenems for treatment of extended spectrum beta lactamase producing *Enterobacteriaceae*. *Sci. Rep.* 11:18. doi: 10.1038/s41598-020-78098-4
- Katoh, K., and Standley, D. M. (2013). MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol. Biol. Evol.* 30, 772–780. doi: 10.1093/molbev/mst010
- Kieffer, N., Royer, G., Decousser, J.-W., Bourrel, A.-S., Palmieri, M., Ortiz De La Rosa, J.-M., et al. (2019). *mcr-9*, an inducible gene encoding an acquired phosphoethanolamine transferase in *Escherichia coli*, and its origin. *Antimicrob. Agents Chemother.* 63:e00965-19. doi: 10.1128/AAC.00965-19
- Koboldt, D. C., Zhang, Q., Larson, D. E., Shen, D., McLellan, M. D., Lin, L., et al. (2012). VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res.* 22, 568–576. doi: 10.1101/gr.129684.111
- Krutova, M., Kalova, A., Nycova, E., Gelbicova, T., Karpiskova, R., Smelikova, E., et al. (2021). The colonisation of Czech travellers and expatriates living in the Czech Republic by colistin-resistant *Enterobacteriaceae* and whole genome characterisation of *E. coli* isolates harbouring the *mcr-1* genes on a plasmid or chromosome: A cross-sectional study. *Travel Med. Infect. Dis.* 39:101914. doi: 10.1016/j.tmaid.2020.101914
- Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923
- Larsen, M. V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R. L., et al. (2012). Multilocus sequence typing of total-genome-sequenced bacteria. *J. Clin. Microbiol.* 50, 1355–1361. doi: 10.1128/jcm.06094-11
- Letunic, I., and Bork, P. (2021). Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. *Nucleic Acids Res.* 49, W293–W296. doi: 10.1093/nar/gkab301
- Li, Y., Dai, X., Zeng, J., Gao, Y., Zhang, Z., and Zhang, L. (2020). Characterization of the global distribution and diversified plasmid reservoirs of the colistin resistance gene *mcr-9*. *Sci. Rep.* 10:8113. doi: 10.1038/s41598-020-65106-w
- Liao, W., Cui, Y., Quan, J., Zhao, D., Han, X., Shi, Q., et al. (2022). High prevalence of colistin resistance and *mcr-9/10* genes in *Enterobacter* spp. in a tertiary hospital over a decade. *Int. J. Antimicrob. Agents* 59:106573. doi: 10.1016/j.ijantimicag.2022.106573
- Lin, Y., Yuan, J., Kolmogorov, M., Shen, M. W., Chaisson, M., and Pevzner, P. A. (2016). Assembly of long error-prone reads using de Bruijn graphs. *Proc. Natl. Acad. Sci.* 113, E8396–E8405. doi: 10.1073/pnas.1604560113
- Marchetti, V. M., Bitar, I., Sarti, M., Fogato, E., Scaltriti, E., Bracchi, C., et al. (2021). Genomic characterization of VIM and MCR co-producers: the first two clinical cases, in Italy. *Diagnostics* 11:79. doi: 10.3390/diagnostics11010079
- Meier-Kolthoff, J. P., Auch, A. F., Klenk, H.-P., and Göker, M. (2013). Genome sequence-based species delimitation with confidence intervals and improved distance functions. *BMC Bioinform.* 14:60. doi: 10.1186/1471-2105-14-60
- Page, A. J., Cummins, C. A., Hunt, M., Wong, V. K., Reuter, S., Holden, M. T. G., et al. (2015). Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 31, 3691–3693. doi: 10.1093/bioinformatics/btv421
- Papagiannitsis, C. C., Študentová, V., Izdebski, R., Oikonomou, O., Pfeifer, Y., Petinaki, E., et al. (2015). Matrix-assisted laser desorption/ionization–time of flight mass spectrometry meropenem hydrolysis assay with NH₄HCO₃, a reliable tool for direct detection of carbapenemase activity. *J. Clin. Microbiol.* 53, 1731–1735. doi: 10.1128/JCM.03094-14
- Price, M. N., Dehal, P. S., and Arkin, A. P. (2010). FastTree 2—approximately maximum-likelihood trees for large alignments. *PLoS One* 5:e9490. doi: 10.1371/journal.pone.0009490
- Prim, N., Turbau, M., Rivera, A., Rodríguez-Navarro, J., Coll, P., and Mirelis, B. (2017). Prevalence of colistin resistance in clinical isolates of *Enterobacteriaceae*: A four-year cross-sectional study. *J. Infect.* 75, 493–498. doi: 10.1016/j.jinf.2017.09.008
- Quiroga, C., Nastro, M., and Di Conza, J. (2019). Current scenario of plasmid-mediated colistin resistance in Latin America. *Rev. Argent. Microbiol.* 51, 93–100. doi: 10.1016/j.ram.2018.05.001
- Rebelo, A. R., Bortolaia, V., Kjeldgaard, J. S., Pedersen, S. K., Leekitcharoenphon, P., Hansen, I. M., et al. (2018). Multiplex PCR for detection of plasmid-mediated colistin resistance determinants, *mcr-1*, *mcr-2*, *mcr-3*, *mcr-4* and *mcr-5* for surveillance purposes. *Euro Surveill.* 23, 17–00672. doi: 10.2807/1560-7917.ES.2018.23.6.17-00672

- Ruan, Z., Sun, Q., Jia, H., Huang, C., Zhou, W., Xie, X., et al. (2019). Emergence of a ST2570 *Klebsiella pneumoniae* isolate carrying *mcr-1* and *blaCTX-M-14* recovered from a bloodstream infection in China. *Clin. Microbiol. Infect.* 25, 916–918. doi: 10.1016/j.cmi.2019.02.005
- Stamatakis, A. (2014). RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312–1313. doi: 10.1093/bioinformatics/btu033
- Tkadlec, J., Kalova, A., Brajerova, M., Gelbicova, T., Karpiskova, R., Smelikova, E., et al. (2021). The intestinal carriage of plasmid-mediated colistin-resistant Enterobacteriaceae in tertiary care settings. *Antibiotics* 10:258. doi: 10.3390/antibiotics10030258
- Tyson, G. H., Li, C., Hsu, C.-H., Ayers, S., Borenstein, S., Mukherjee, S., et al. (2020). The *mcr-9* gene of *Salmonella* and *Escherichia coli* is not associated with colistin resistance in the United States. *Antimicrob. Agents Chemother.* 64:e00573-20. doi: 10.1128/AAC.00573-20
- Viñes, J., Cuscó, A., Napp, S., Alvarez, J., Saez-Llorente, J. L., Rosàs-Rodoreda, M., et al. (2021). Transmission of similar *mcr-1* carrying plasmids among different *Escherichia coli* lineages isolated from livestock and the farmer. *Antibiotics* 10:313. doi: 10.3390/antibiotics10030313
- Walker, B. J., Abeel, T., Shea, T., Priest, M., Abouelliel, A., Sakthikumar, S., et al. (2014). Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. *PLoS One* 9:e112963. doi: 10.1371/journal.pone.0112963
- Wang, C., Feng, Y., Liu, L., Wei, L., Kang, M., and Zong, Z. (2020). Identification of novel mobile colistin resistance gene *mcr-10*. *Emerg. Microbes Infect.* 9, 508–516. doi: 10.1080/22221751.2020.1732231
- Wang, X., Wang, Y., Zhou, Y., Li, J., Yin, W., Wang, S., et al. (2018). Emergence of a novel mobile colistin resistance gene, *mcr-8*, in NDM-producing *Klebsiella pneumoniae*. *Emerg. Microbes Infect.* 7, 1–9. doi: 10.1038/s41426-018-0124-z
- Wang, Y., Xu, C., Zhang, R., Chen, Y., Shen, Y., Hu, F., et al. (2020). Changes in colistin resistance and *mcr-1* abundance in *Escherichia coli* of animal and human origins following the ban of colistin-positive additives in China: an epidemiological comparative study. *Lancet Infect. Dis.* 20, 1161–1171. doi: 10.1016/s1473-3099(20)30149-3
- Wick, R. R., Judd, L. M., Gorrie, C. L., and Holt, K. E. (2017). Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. *PLoS Comput. Biol.* 13:e1005595. doi: 10.1371/journal.pcbi.1005595
- Xavier, B. B., Lammens, C., Butaye, P., Goossens, H., and Malhotra-Kumar, S. (2016). Complete sequence of an IncFII plasmid harbouring the colistin resistance gene *mcr-1* isolated from Belgian pig farms. *J. Antimicrob. Chemother.* 71, 2342–2344. doi: 10.1093/jac/dkw191
- Yilmaz, G. R., Dizbay, M., Guven, T., Pullukcu, H., Tasbakan, M., Guzel, O. T., et al. (2016). Risk factors for infection with colistin-resistant gram-negative microorganisms: a multicenter study. *Ann. Saudi Med.* 36, 216–222. doi: 10.5144/0256-4947.2016.216
- Yoon, S. H., Ha, S. M., Kwon, S., Lim, J., Kim, Y., Seo, H., et al. (2017). Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. *Int. J. Syst. Evol. Microbiol.* 67:1613. doi: 10.1099/ijsem.0.001755
- Zankari, E., Allesøe, R., Joensen, K. G., Cavaco, L. M., Lund, O., and Aarestrup, F. M. (2017). PointFinder: a novel web tool for WGS-based detection of antimicrobial resistance associated with chromosomal point mutations in bacterial pathogens. *J. Antimicrob. Chemother.* 72, 2764–2768. doi: 10.1093/jac/dkx217
- Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., et al. (2012). Identification of acquired antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 2640–2644. doi: 10.1093/jac/dks261
- Zelendova, M., Papagiannitsis, C. C., Valcek, A., Medvecky, M., Bitar, I., Hrabak, J., et al. (2021). Characterization of the complete nucleotide sequences of *mcr-1*-encoding plasmids from *Enterobacteriales* isolates in retail raw meat products from the Czech Republic. *Front. Microbiol.* 11:604067. doi: 10.3389/fmicb.2020.604067
- Zhang, Z., Tian, X., and Shi, C. (2022). Global Spread of MCR-Producing *Salmonella enterica* Isolates. *Antibiotics* 11:998. doi: 10.3390/antibiotics11080998
- Zhu, W., Lawsin, A., Lindsey, R. L., Batra, D., Knipe, K., Yoo, B. B., et al. (2019). Conjugal transfer, whole-genome sequencing, and plasmid analysis of four *mcr-1*-bearing isolates from U.S. patients. *Antimicrob. Agents Chemotherapy* 63:e02417-18. doi: 10.1128/AAC.02417-18
- Zurfluh, K., Nüesch-Inderbinen, M., Klumpp, J., Poirer, L., Nordmann, P., and Stephan, R. (2017). Key features of *mcr-1*-bearing plasmids from *Escherichia coli* isolated from humans and food. *Antimicrob. Resistan. Infect. Control* 6:91. doi: 10.1186/s13756-017-0250-8